ORIGINAL ARTICLE

Prognostic Impact of Tumor Cell Programmed Death Ligand 1 Expression and Immune Cell Infiltration in NSCLC

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ABSTRACT

Introduction: Infiltration of T and B/plasma cells has been linked to NSCLC prognosis, but this has not been thoroughly investigated in relation to the expression of programmed death ligand 1 (PD-L1). Here, we determine the association of lymphocytes and PD-L1 with overall survival (OS) in two retrospective cohorts of operated NSCLC patients who were not treated with checkpoint inhibitors targeting the programmed death 1/PD-L1 axis. Moreover, we evaluate how PD-L1 positivity and clinicopathologic factors affect the prognostic association of lymphocytes.

Methods: Cluster of differentiation (CD) 3 (CD3)-, CD8-, CD4-, forkhead box P3 (FOXP3)-, CD20-, CD79A-, and immunoglobulin kappa constant (IGKC)-positive immune cells, and tumor PD-L1 positivity, were determined by immunohistochemistry on tissue microarrays (n = 705). Affymetrix data was analyzed for a patient subset, and supplemented with publicly available transcriptomics data (N = 1724). Associations with OS were assessed by Kaplan-Meier plots and uni- and multivariate Cox regression.

Results: Higher levels of T and B plasma cells were associated with longer OS ($p = 0.004$ and $p < 0.001$, for CD8 and IGKC, respectively). Highly proliferative tumors with few lymphocytes had the worst outcome. No association of PD-L1 positivity with OS was observed in a nonstratified patient population; however, a significant association with shorter OS was observed in never-smokers ($p = 0.009$ and $p = 0.002$, 5% and 50% cutoff). Lymphocyte infiltration was not associated with OS in PD-L1–positive tumors (50% cutoff). The prognostic association of lymphocyte infiltration also depended on the patients' smoking history and histologic subtype.

Conclusions: Proliferation, PD-L1 status, smoking history, and histology should be considered if lymphocyte infiltration is to be used as a prognostic biomarker.

Keywords: Lymphocyte; Prognosis; Adenocarcinoma; Squamous cell carcinoma; Ki67

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Introduction

An increased number of tumor-infiltrating lymphocytes in primary tumor tissue has been linked to better prognosis in several cancer types, including NSCLC.[1-4] With the success of programmed death protein 1 (PD-1) and programmed death ligand 1 (PD-L1) targeting therapies, immune-inhibitory factors have become another main focus in NSCLC.[5-9] Expression of PD-L1 on the tumor cell surface permits the interaction with PD-1 on T cells and inhibition of the local antitumor immune response.[10] Therapeutically blocking the interaction between PD-L1 and PD-1 allows T cell reactivation, and patients with advanced NSCLC who were treated with a PD-1 or PD-L1 inhibitor have shown a survival benefit, with PD-L1 positivity used as a predictive marker of therapy response.[7-9,11,12] However, the prognostic impact of PD-L1 expression in NSCLC patients who did not receive treatment with checkpoint inhibitors targeting the PD-1/PD-L1 axis is unclear because the results of published studies so far are inconsistent.[13] In this patient population, it has been reported that PD-L1 positivity is associated with better as well as worse outcome, or no association with outcome was observed.[14-21] Methodological differences, such as the use of different antibody clones, immunohistochemistry (IHC) protocols, and cutoffs for PD-L1 positivity is likely to contribute to these dissimilar findings, but they may also be due to differences in the distribution of demographic or clinicopathologic parameters in the evaluated patient cohorts, or to the levels of infiltrating immune cells in the corresponding tumors. In the present investigation, we aimed to (1) determine the association of lymphocyte infiltration and PD-L1 status with overall survival (OS), and (2) evaluate how PD-L1 positivity and clinicopathologic factors affect the prognostic association of lymphocyte infiltration in NSCLC patients not treated with PD-1/PD-L1 inhibitors.

Materials and Methods

Patients and Data

The Uppsala-I and Uppsala-II cohorts included NSCLC patients operated at Uppsala University Hospital 1995-2005 (n = 353) and 2006-2010 (n = 352), respectively.[22,23] Neoadjuvantly treated patients were excluded. Clinicopathologic information and survival follow-up were available from the population-based Uppsala-Örebro Lung Cancer Register, and by evaluation of patient charts and diagnostic pathology reports (Table 1). ALK receptor tyrosine kinase (ALK) rearrangement and EGFR mutation status were reported previously for 611 and 95 of these patients, respectively.[23,24] Formalin-fixed paraffin-embedded tumor tissue blocks were available from the Department of Pathology at Uppsala University Hospital. The analysis of human tissue and corresponding clinicopathologic information was approved by the Uppsala Regional Ethical Review Board (reference number 2006/325 and 2012/532).

For Uppsala-I, Affymetrix HG-U133 Plus 2.0 microarray data was available for 194 patients (Gene Expression Omnibus accession number GSE37745), of which 189 overlapped with those for which formalin-fixed paraffin-embedded tissue was available.[22] For validation purposes, eight additional Affymetrix HG-U133A or Plus 2.0 NSCLC datasets, with available information on OS, were downloaded from Gene Expression Omnibus: GSE14814, GSE19188, GSE30219, GSE31210, GSE3141, GSE4573, GSE50081, or from the website of the Director's Challenge Consortium for the Molecular Classification of Lung Adenocarcinoma (Supplementary Table 1). Frozen robust multi-array average was used for normalization of the Affymetrix array data, except for GSE4573 and GSE3141 for which only MAS5 normalized data was available.[25]

IHC

Tissue microarrays (TMA) were produced with duplicate tissue cores (1 mm in diameter) acquired from each tumor block using a manual tissue arrayer (MTA-1, Beecher Instruments, Sun Prairie, California). IHC was performed with antibodies towards Cluster of differentiation (CD) 3 (CD3), CD8, CD4, forkhead box P3 (FOXP3), CD20, CD79A, immunoglobulin kappa constant (IGKC), PD-L1, and Ki67, as detailed in the Supplementary Methods. The stained TMA slides were scanned (original magnification ×20) using the Aperio ScanScope XT (Aperio, Vista, California) whole-slide scanner and the digital images were imported into the openly available software ImageScope (Aperio). For each antibody, one semiquantitative score was given for the duplicate cores from each patient. PD-L1 expression was assessed on tumor cells, taking only membranous positivity into account, as: (0) negative: less than 5%, (1) low: 5% to 25%, (2) moderate: 26% to 50%, and (3) high: 51% to 100% positive tumor cells; for subsequent analysis dichotomized as negative (0) versus positive (1-3; cutoff positivity 5% or greater), and alternatively negative (0-2) versus positive (3; cutoff positivity 50% or greater). Stromal lymphocyte infiltration was for CD3, CD4, CD8, FOXP3, CD20, CD79A, and IGKC assessed as: (0) no positive cells or few dispersed positive cells, (1) infiltration of less than 25% of the stromal area or sparsely dispersed positive cells across the entire core area, (2) infiltration of 25% to 49% of the stromal area, and (3) infiltration 50% or greater of the stromal area; for the analysis dichotomized as low (0-1) versus high (2-3), alternatively low (0-2) versus high (3), infiltration. IHC scoring is further described and shown in Supplementary Figure 1. The IHC evaluation of CD20 and IGKC has been reported previously for the Uppsala-I

Table 1. Distribution of Clinicopathologic Characteristics in the Two NSCLC Tissue Microarray Cohorts

	Uppsala-I		Uppsala-II		
Characteristics	n	%	n	%	*p* Value
All patients	353	100	352	100	
Sex					
Male	188	53.3	171	48.6	0.228
Female	165	46.7	181	51.4	
Age at diagnosis, years					
<70	222	62.9	215	61.1	0.698
≥70	131	37.1	136	38.6	
Missing	0	0.0	1	0.3	
Smoking history					
Current/former smoker	317	89.8	310	88.1	0.469
Never smoker	35	9.9	42	11.9	
Missing	1	0.3	0	0.0	
Performance status					
0	186	52.7	213	60.5	0.040[a]
1	133	37.7	136	38.6	
2	28	7.9	3	0.9	
3	5	1.4	0	0.0	
4	1	0.3	0	0.0	
pTNM-stage					
IA	89	25.2	144	40.9	0.207[b]
IB	149	42.2	77	21.9	
IIA	12	3.4	41	11.6	
IIB	42	11.9	34	9.7	
IIIA	34	9.6	49	13.9	
IIIB	17	4.8	0	0.0	
IV	10	2.8	7	2.0	
Histology					
Adenocarcinoma	193	54.7	208	59.1	0.241[c]
Squamous cell carcinoma	118	33.4	103	29.3	
Large cell carcinoma	40	11.3	30	8.5	
Other	1	0.3	11[d]	3.2	
Missing	1	0.3			
Proliferation					
Ki67 low	101	28.6	126	35.8	0.052[e]
Ki67 high	248	70.3	223	63.4	
Missing	4	1.1	3	0.9	
Overall survival					
Dead (5 year)	241 (198)	68.7 (54.6)	195 (180)	55.4 (51.1)	0.001[f]
Alive at last follow-up (5 year)	112 (155)	31.3 (45.4)	156 (171)	44.3 (48.6)	
Missing (5 year)	0 (0)	0.0 (0.0)	1 (1)	0.3 (0.3)	
Follow-up days, mean (median)	1846 (1546)		1532 (1626)		

p values are from the Fisher's exact test.
[a]Performance status 0 vs. 1-4.
[b]Stage I vs. stage II-IV.
[c]Adenocarcinoma vs. squamous cell carcinoma.
[d]Adenosquamous n = 8; sarcomatoid n = 3.
[e]Immunohistochemistry score low <20% and high ≥20% Ki67-positive nuclei.
[f]$p = 0.227$ when including restricted to the first 5 years after surgery.

cohort, and has in the present investigation been supplemented by stainings of the Uppsala-II cohort.[4]

Statistical Analysis

The statistical programming language R version 3.4.1 and IBM SPSS Statistics Version 25 (IBM, Armonk, NY) were used. *p* values were two-sided and 0.05 was used as the level of significance. OS was defined by the time interval between date of diagnosis and date of death or last follow-up. Patients with a survival time shorter than 1 week were excluded. The Kaplan-Meier method was used to plot the survival rates and they were compared with the log-rank test. Uni- and multivariate Cox proportional hazards regression models were used to test

associations with OS, and results were presented with hazard ratios (HRs), 95% confidence intervals (CIs), and p values. The multivariate models were fitted with inclusion of the following covariables: IHC score (low versus high), sex (male versus female), age (<70 versus ≥ 70 years), performance status according to Eastern Cooperative Oncology Group (0 versus 1-4), stage (I versus II-IV), histology (adenocarcinoma versus non-adenocarcinoma), smoking history (never versus current/former), and proliferation (Ki67 low, $<20\%$ versus high, $\geq 20\%$). Meta-analyses were performed based on parameter estimates (log HR) in the univariate Cox model and their corresponding standard errors, using the R package meta 4.8-1. Inverse variance weighting was used to combine single estimates into one pooled estimate. The significance of the overall effect was assessed by the p value of the random effects model, and the results were visualized by forest plots. The Fisher's exact test was applied to test associations between two categorical variables. The Pearson correlation coefficient was used to assess the correlation between gene expression and IHC scores, and the corresponding p values were determined by one-way analysis of variance. The Spearman rank correlation coefficient was used to assess genome-wide correlations of gene expression. Genes with a false discovery rate adjusted p less than 0.01 and a Spearman correlation coefficient rho greater than 0.4 were used as input for Gene Ontology (GO) enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery 6.7.[26,27]

Results

Patient Characteristics

The distribution of patients according to sex, age, smoking history, stage, histology, and proliferation did not differ significantly between the two TMA cohorts, Uppsala-I and Uppsala-II; however, a higher number of patients with lower performance status was observed in Uppsala-II ($p = 0.040$, Fisher's exact test) (Table 1). Also, fewer deaths were observed in Uppsala-II ($p = 0.001$), but this difference was not significant when compensating for the shorter follow-up time by only taking events during the first 5 years after diagnosis into account ($p = 0.227$).

A significant association with longer OS was observed in both Uppsala-I and Uppsala-II for lower stage ($p = 0.001$ and $p < 0.001$, respectively, log-rank test), better performance status (both $p < 0.001$), and lower proliferation index ($p = 0.027$ and $p = 0.005$) (Supplementary Fig. 2). A significant association with better outcome was also found for younger patients in Uppsala-I ($p < 0.001$) and females in Uppsala-II ($p = 0.028$); whereas, histology, smoking history, ALK rearrangement, and EGFR mutation status were not associated with OS (Supplementary Fig. 2).

Lymphocyte Infiltration is Associated With Higher Tumor Cell Proliferation and Better OS

The level of lymphocyte infiltration was evaluated by IHC using antibodies toward CD20 (B cells), CD79A (B and plasma cells), IGKC (plasma cells), CD3 (T cells), CD8 (cytotoxic T cells), CD4 (T helper cells), and FOXP3 (regulatory T cells). High infiltration (IHC score 2-3) of CD20-, CD79A-, and IGKC-positive cells (Fig. 1*A*; Supplementary Figs. 1*A-C*) were observed in 24.6%, 53.1%, and 29.7%, respectively. For CD3-, CD8-, CD4-, and FOXP3-positive cells (Fig. 1*A*; Supplementary Figs. 1*D-G*), the corresponding percentages were 64.7%, 50.4%, 48.4%, and 24.4%. The distribution of the IHC scores in the Uppsala-I and Uppsala-II cohorts were similar (Supplementary Figs. 1*A-G*), and since the differences between the cohorts concerning the distribution of clinicopathologic factors were also minor (Table 1), the two cohorts were combined in the subsequent analyses (total n = 705).

A significant correlation with higher proliferation was observed for higher IHC scores, dichotomized as either 0-1 versus 2-3 or 0-2 versus 3, for all antibodies; with better performance status for CD8; with lower stage for CD20, CD79A, CD4, and FOXP3; with non-adenocarcinoma histology for IGKC, CD4, and FOXP3; and with a history of smoking for IGKC (Supplementary Table 2*A-B*). The pan-T cell marker CD3 correlated significantly with CD8, CD4, and FOXP3, and significant correlations were also found between the B/plasma cell markers CD20, CD79A, and IGKC; moreover, low levels of cells positive for the T cell markers correlated with low levels of the B/plasma cell markers for all combinations and for both dichotomizations (all $p < 0.01$, Fisher's exact test, data not shown). This indicates that lymphocyte infiltration generally is not restricted to cells of either the T or B cell lineage, or a specific subpopulation (of those analyzed), but rather that the manifestation of one cell type coincides with that of other.

A significant association with longer OS for higher lymphocyte levels (IHC score 0-1 versus 2-3) was observed for CD3 ($p = 0.008$), CD8 ($p = 0.004$), CD4 ($p = 0.005$), CD20 ($p = 0.042$), CD79A ($p = 0.004$), and IGKC ($p < 0.001$), but not for FOXP3 ($p = 0.060$) (Fig. 1*A*) (results for the separate cohorts are shown in Supplementary Fig. 3*A*). Significant associations were also obtained when an alternative cutoff for high-level infiltration was used (IHC score 0-2 versus 3), with the exception of CD20 (Supplementary Fig. 3*B*), and for CD3, CD8, CD4, CD79A, and IGKC when the IHC scores were included without prior dichotomization (Supplementary Fig. 3*C*), indicating that the findings do not depend exclusively on the cutoff for dichotomization of the IHC scores. Also, the semiquantitative IHC scores generally

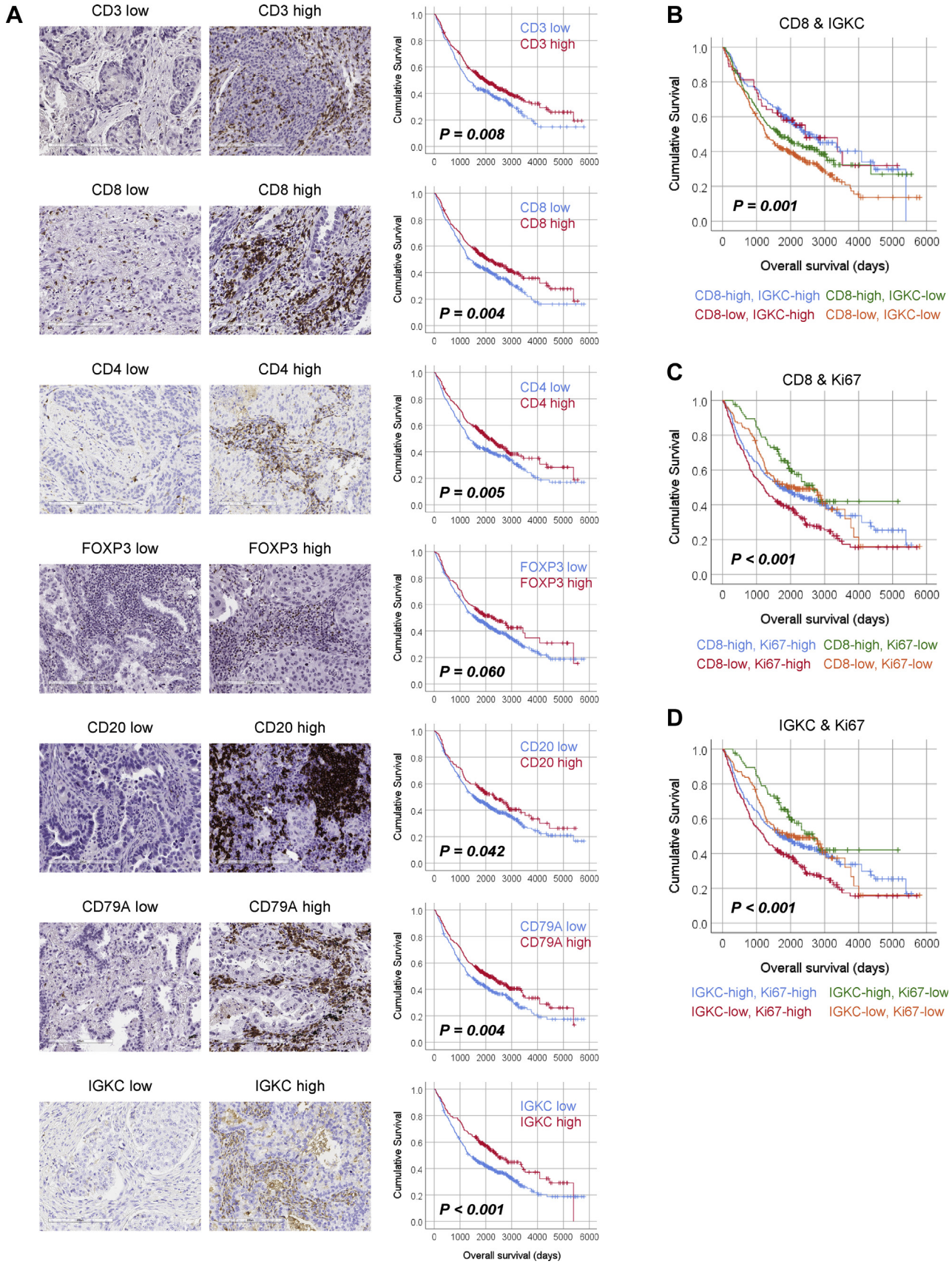

Figure 1. Prognostic associations of immune cell infiltration. (*A*) Immunohistochemistry (IHC) scores for high and low levels of infiltrating lymphocytes in the tumor tissue microenvironment (*left panel*) and Kaplan-Meier plots contrasting low (IHC score 0-1) versus high (IHC score 2-3) level (*right panel*) of CD3, CD8, CD4, FOXP3, CD20, CD79A, and IGKC-positive cells. (*B*) Kaplan-Meier plot for combinations of CD8 and IGKC IHC scores. (*C*) Kaplan-Meier plot for combinations of CD8 and Ki67 IHC scores. (*D*) Kaplan-Meier plot for combinations of IGKC and Ki67 IHC scores. *p* value from the log rank test.

correlated well with corresponding transcript levels for the subset of patients for which gene expression information was additionally available, except for CD4 (Fig. 2*A*). Using a meta-analysis approach, including in total nine Affymetrix datasets, a significant association with OS for the overall effect was observed on the RNA level for the genes encoding CD20 (*MS4A1*; membrane spanning 4-Domains A1), CD79A (*CD79A*, CD79a molecule), and CD3 (*CD3D*, CD3d molecule) (Fig. 2*B*), in support of the IHC-based findings.

Next, the concurrent presence of T cells, as well as B and plasma cells, in the tumor microenvironment, as well as tumor proliferation, were considered. Tumors with low infiltration of both CD8- and IGKC-positive cells had shorter OS than tumors with a low level of either cell type alone (Fig. 1*B*; Supplementary Fig. 3*D*). Patients with highly proliferative tumors and a low level of CD8- or IGKC-positive cells, respectively, had the worst outcome (Figs. 1*C* and *D*; Supplementary Fig. 3*D*). In a multivariate analysis, also including clinicopathologic factors and proliferation, a significantly worse survival outcome was observed for patients with CD8-low/IGKC-low (HR: 1.65, 95% CI: 1.26–2.17, $p < 0.001$) compared with CD8-high/IGKC-high IHC scores (Table 2).

PD-L1 Expression Correlates With T and B Cell Infiltration

Both proliferation and concurrent high levels of both T cells and plasma cells appeared to be important for the effect of lymphocyte infiltration on OS, but the possible influence of immune-inhibitory factors is also of interest. In the present study, membranous tumor cell PD-L1 positivity (Fig. 3*A*) was observed in 34.1% and 39.5% ($\geq$ 5% cutoff for positivity), or 15.0% and 21.6% ($\geq$ 50% cutoff), in the two cohorts, respectively (Fig. 3*B*; Supplementary Fig. 1*H*). PD-L1 positivity correlated with nonadenocarcinoma histology and high proliferation, as well as with a higher level of lymphocytes, regardless of the used cutoff (Supplementary Table 3*A*-*B*). Also, the semiquantitative PD-L1 IHC scores correlated with the expression levels of the gene encoding PD-L1 (*CD274*) (Affymetrix probeset: 223834_at) in the subset of the Uppsala-I cohort where both data types were available (Fig. 3*C*). Based on the genes with a significant positive correlation with *CD274* (false discovery rate adjusted $p < 0.01$) and a Spearman correlation coefficient rho 0.4 or greater (Supplementary Table 4*A*), top enriched biological process GOs included, for instance, "regulation of interferon-gamma-mediated signaling pathway" (GO: 0060334), "regulation of inflammatory response" (GO: 0050727), "apoptotic process" (GO: 0006915), and "regulation of cell proliferation" (GO: 0042127) (Supplementary Table 4*B*). This further supports previous reports that PD-L1 positivity is associated with inflammation and the presence of immune cells in the primary tumor.[14,19]

Association of PD-L1 With OS

Next, the association of PD-L1 tumor cell positivity (assessed by IHC) with OS was evaluated. No association between PD-L1 and OS was observed in the combined cohort, regardless of whether 5% or greater ($p = 0.729$) or 50% or greater ($p = 0.654$) was used as the cutoff for positivity (Fig. 3*D*). The same was observed for *CD274* expression (223834_at), analyzed as a continuous variable, in a meta-analysis of publicly available gene expression microarray datasets, including GSE37745 which corresponded to a subset of the patients of the Uppsala-I cohort (Fig. 3*E*). In the combined TMA cohort, PD-L1 was also not associated with OS when analyzed stratified according to histology, sex, stage, performance status, proliferation index, age, ALK status, EGFR mutation status, or the level of lymphocyte infiltration, as assessed by the dichotomized IHC scores for CD8 or IGKC (Supplementary Figs. 4*A*-*C*). However, a significant association of PD-L1 positivity with shorter OS was observed in never-smokers ($p = 0.009$ and $P = 0.002$ for $\geq$5% and $\geq$50% cutoffs, respectively) (Fig. 3*F*); whereas, PD-L1 was not associated with OS in current and former smokers ($p = 0.645$ and $p = 0.770$). In a multivariate analysis, the association with worse outcome, however, reached only borderline significance in never-smokers (univariate: HR: 3.454, 95% CI: 1.487–8.021, $p = 0.004$; multivariate: HR: 2.484, 95% CI: 0.976–6.322, $p = 0.056$) (Supplementary Table 5).

In the Affymetrix dataset GSE37745, which corresponds to a subset of the patients of the Uppsala-I TMA cohort, a significant association with shorter OS in never-smokers was also observed for *CD274* (223834_at) in a univariate Cox analysis (HR: 9.39, 95% CI: 1.08–81.9, $p = 0.04$; n = 15), despite the limited number of never-smokers, but not in former/current smokers (Supplementary Fig. 5*A*). Extending the analysis to include also the two further public Affymetrix datasets with available information on smoking history in a meta-analysis, this finding was supported for one probeset (223834_at; $p < 0.001$); whereas, a nonsignificant *p* value for the overall effect was obtained for the other probeset (227458_at; $p = 0.0752$) (Supplementary Fig. 5*B*).

PD-L1, Smoking History, and Histology and the Prognostic Influence of Lymphocyte Infiltration

As a final point, we evaluated if the prognostic impact of lymphocyte infiltration, here focusing on CD8 and IGKC, depended on PD-L1 status. Because of the explorative nature of the following subgroup analyses, the presented results must, however, be interpreted with caution. In PD-L1–negative tumors, a

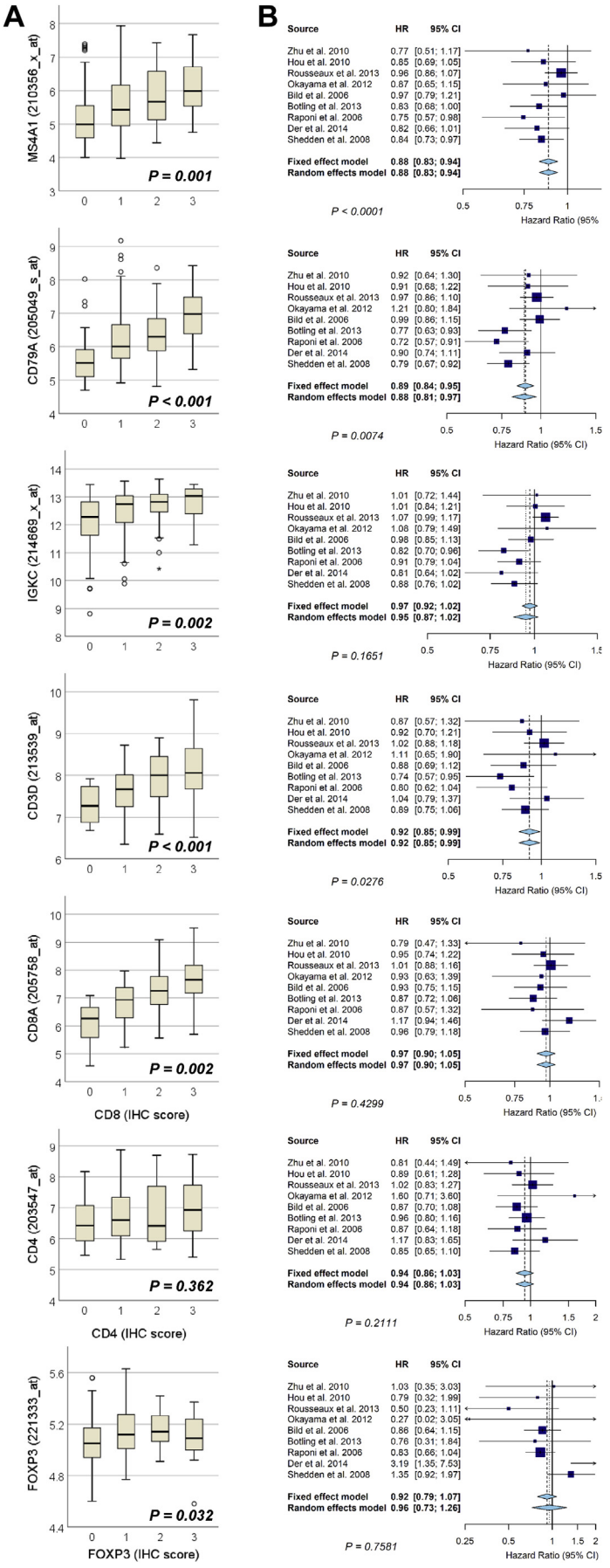
A
B
MS4A1 (210356_x_at)
P = 0.001
CD79A (205049_s_at)
P < 0.001
IGKC (214669_x_at)
P = 0.002
CD3D (213539_at)
P < 0.001
CD8A (205758_at)
P = 0.002
CD8 (IHC score)
CD4 (203547_at)
P = 0.362
CD4 (IHC score)
FOXP3 (221333_at)
P = 0.032
FOXP3 (IHC score)
Source HR 95% CI
Zhu et al. 2010 0.77 [0.51; 1.17]
Hou et al. 2010 0.85 [0.69; 1.05]
Rousseaux et al. 2013 0.96 [0.86; 1.07]
Okayama et al. 2012 0.87 [0.65; 1.15]
Bild et al. 2006 0.97 [0.79; 1.21]
Botling et al. 2013 0.83 [0.68; 1.00]
Raponi et al. 2006 0.75 [0.57; 0.98]
Der et al. 2014 0.82 [0.66; 1.01]
Shedden et al. 2008 0.84 [0.73; 0.97]
Fixed effect model 0.88 [0.83; 0.94]
Random effects model 0.88 [0.83; 0.94]
P < 0.0001
Hazard Ratio (95%
Source HR 95% CI
Zhu et al. 2010 0.92 [0.64; 1.30]
Hou et al. 2010 0.91 [0.68; 1.22]
Rousseaux et al. 2013 0.97 [0.86; 1.10]
Okayama et al. 2012 1.21 [0.80; 1.84]
Bild et al. 2006 0.99 [0.86; 1.15]
Botling et al. 2013 0.77 [0.63; 0.93]
Raponi et al. 2006 0.72 [0.57; 0.91]
Der et al. 2014 0.90 [0.74; 1.11]
Shedden et al. 2008 0.79 [0.67; 0.92]
Fixed effect model 0.89 [0.84; 0.95]
Random effects model 0.88 [0.81; 0.97]
P = 0.0074
Hazard Ratio (95% CI)
Source HR 95% CI
Zhu et al. 2010 1.01 [0.72; 1.44]
Hou et al. 2010 1.01 [0.84; 1.21]
Rousseaux et al. 2013 1.07 [0.99; 1.17]
Okayama et al. 2012 1.08 [0.79; 1.49]
Bild et al. 2006 0.98 [0.85; 1.13]
Botling et al. 2013 0.82 [0.70; 0.96]
Raponi et al. 2006 0.91 [0.79; 1.04]
Der et al. 2014 0.81 [0.64; 1.02]
Shedden et al. 2008 0.88 [0.76; 1.02]
Fixed effect model 0.97 [0.92; 1.02]
Random effects model 0.95 [0.87; 1.02]
P = 0.1651
Hazard Ratio (95% CI)
Source HR 95% CI
Zhu et al. 2010 0.87 [0.57; 1.32]
Hou et al. 2010 0.92 [0.70; 1.21]
Rousseaux et al. 2013 1.02 [0.88; 1.18]
Okayama et al. 2012 1.11 [0.65; 1.90]
Bild et al. 2006 0.88 [0.69; 1.12]
Botling et al. 2013 0.74 [0.57; 0.95]
Raponi et al. 2006 0.80 [0.62; 1.04]
Der et al. 2014 1.04 [0.79; 1.37]
Shedden et al. 2008 0.89 [0.75; 1.06]
Fixed effect model 0.92 [0.85; 0.99]
Random effects model 0.92 [0.85; 0.99]
P = 0.0276
Hazard Ratio (95% CI)
Source HR 95% CI
Zhu et al. 2010 0.79 [0.47; 1.33]
Hou et al. 2010 0.95 [0.74; 1.22]
Rousseaux et al. 2013 1.01 [0.88; 1.16]
Okayama et al. 2012 0.93 [0.63; 1.39]
Bild et al. 2006 0.93 [0.75; 1.15]
Botling et al. 2013 0.87 [0.72; 1.06]
Raponi et al. 2006 0.87 [0.57; 1.32]
Der et al. 2014 1.17 [0.94; 1.46]
Shedden et al. 2008 0.96 [0.79; 1.18]
Fixed effect model 0.97 [0.90; 1.05]
Random effects model 0.97 [0.90; 1.05]
P = 0.4299
Hazard Ratio (95% CI)
Source HR 95% CI
Zhu et al. 2010 0.81 [0.44; 1.49]
Hou et al. 2010 0.89 [0.61; 1.28]
Rousseaux et al. 2013 1.02 [0.83; 1.27]
Okayama et al. 2012 1.60 [0.71; 3.60]
Bild et al. 2006 0.87 [0.70; 1.08]
Botling et al. 2013 0.96 [0.80; 1.16]
Raponi et al. 2006 0.87 [0.64; 1.18]
Der et al. 2014 1.17 [0.83; 1.65]
Shedden et al. 2008 0.85 [0.65; 1.10]
Fixed effect model 0.94 [0.86; 1.03]
Random effects model 0.94 [0.86; 1.03]
P = 0.2111
Hazard Ratio (95% CI)
Source HR 95% CI
Zhu et al. 2010 1.03 [0.35; 3.03]
Hou et al. 2010 0.79 [0.32; 1.99]
Rousseaux et al. 2013 0.50 [0.23; 1.11]
Okayama et al. 2012 0.27 [0.02; 3.05]
Bild et al. 2006 0.86 [0.64; 1.15]
Botling et al. 2013 0.76 [0.31; 1.84]
Raponi et al. 2006 0.83 [0.66; 1.04]
Der et al. 2014 3.19 [1.35; 7.53]
Shedden et al. 2008 1.35 [0.92; 1.97]
Fixed effect model 0.92 [0.79; 1.07]
Random effects model 0.96 [0.73; 1.26]
P = 0.7581
Hazard Ratio (95% CI)

Table 2. Uni- and Multivariate Cox Analysis of the Combined Cohort, Including a Combined CD8-IGKC Immunohistochemistry Score

	Univariate				Multivariate			
		95% CI				95% CI		
	HR	Lower	Higher	*p*	HR	Lower	Higher	*p*
Histology								
Non-adenocarcinoma	1.000				1.000			
Adenocarcinoma	0.996	0.824	1.204	0.965	1.461	1.174	1.819	0.001
Stage								
I	1.000				1.000			
II-IV	1.809	1.495	2.189	<0.001	1.640	1.345	1.999	<0.001
Performance status								
0	1.000				1.000			
1-4	1.767	1.464	2.134	<0.001	1.600	1.315	1.948	<0.001
Age, years								
<70	1.000				1.000			
≥70	1.393	1.151	1.685	0.001	1.286	1.056	1.568	0.013
Sex								
Male	1.000				1.000			
Female	0.807	0.669	0.975	0.026	0.816	0.669	0.997	0.046
Smoking								
Never	1.000				1.000			
Current/former	1.283	0.933	1.763	0.125	1.142	0.810	1.612	0.448
Proliferation								
Ki67 low	1.000				1.000			
Ki67 high	1.460	1.182	1.802	<0.001	1.669	1.304	2.136	<0.001
CD8/IGKC[a]								
CD8 high; IGKC high	1.000				1.000			
CD8 low; IGKC high	1.020	0.659	1.579	0.929	0.969	0.625	1.503	0.888
CD8 high; IGKC low	1.302	0.977	1.737	0.072	1.356	1.014	1.812	0.040
CD8 low; IGKC low	1.654	1.273	2.148	<0.001	1.652	1.261	2.165	<0.001

[a]CD8 and IGKC IHC scores dichotomized as low 0-1 vs. high 2-3.
CI, confidence interval; HR, hazard ratio.

higher level of CD8-positive cells was significantly associated with longer OS, and this was observed regardless of the cutoff used to define PD-L1 positivity (Fig. 4*A*, *left panel*). On the contrary, no significant association with OS was observed for CD8 in PD-L1–positive tumors, when positivity was defined as 50% or greater positive tumor cells (Fig. 4*A*, *right panel*). The same was observed for the level of plasma cells (Fig. 4*B*). However, it should be considered that the number of PD-L1–positive tumors (n = 121) was smaller compared to PD-L1–negative tumors (n = 536), and it cannot be excluded that the lack of survival association is due to the smaller sample size.

Thereafter, the influence of the patients' smoking history and histologic subtype on the prognostic impact of CD8-positive T cell infiltration was evaluated. The level of CD8-positive T cell infiltration was not associated with prognosis in never-smokers, in contrast to current/former smokers (Fig. 4*C*). However, the never-smokers in this cohort were characterized by a higher proportion of slowly proliferating tumors ($p < 0.001$, Fisher's exact test). CD8-positive T cell infiltration showed a significant association with better prognosis in adenocarcinomas but not in nonadenocarcinomas (Fig. 4*D*). An analysis stratified according to both histologic subtype and smoking history is challenging because of the low case number of the never-smoker patient subset. Nevertheless, in never-smoking non-adenocarcinomas (i.e., squamous cell carcinomas and large cell carcinomas) the beneficial prognostic association of T cell infiltration was not observed; rather the opposite was seen (Fig. 4*E*) while there was no clear

Figure 2. Transcript-level validation of immunohistochemistry (IHC) scores and confirmation of prognostic associations in publicly available datasets. (*A*) IHC score versus transcript level (Affymetrix probeset) for CD20 (210356_x_at, *MS4A1*), CD79A (205049_s_at, *CD79A*), IGKC (214669_x_at, *IGKC*), CD3 (213539_at, *CD3D*), CD8 (205758_at, *CD8A*), CD4 (203547_at), and FOXP3 (221333_at). *p* value from the one-way analysis of variance. (*B*) Association with overall survival for immune cell type-specific transcript levels in a meta-analysis of Affymetrix NSCLC datasets. HR, hazard ratio; 95% CI, 95% confidence interval; *p* value shown of the random effects model.

difference due to histologic subtype in current/former smokers (Fig. 4*F*). This opposite pattern in non-adenocarcinoma never-smokers was not observed for IGKC (Supplementary Figs. 6*A-D*).

Discussion

The importance of the antitumor immune response in relation to outcome has been shown in several cancer types, including NSCLC, using gene expression–based immune signatures, as well as using IHC with antibodies that recognize different subpopulations of immune cells.[1-3,28] In NSCLC, the infiltration of CD4-positive helper and CD8-positive cytotoxic T cells was linked to better outcome and FOXP3-positive regulatory T cells to worse outcome.[29-31] Only few studies evaluated markers associated with the presence of B or plasma cells.[4,29,30,32,33] Using IHC, the present study confirms that a high level of infiltrating lymphocytes of the T as well as B cell lineage is associated with better outcome in NSCLC. A low level of lymphocyte infiltration in combination with high proliferation was associated with the worst outcome. Moreover, a significant association was also observed on the RNA level for *MS4A1* (CD20), *CD79A*, and *CD3D* in a meta-analysis of a large

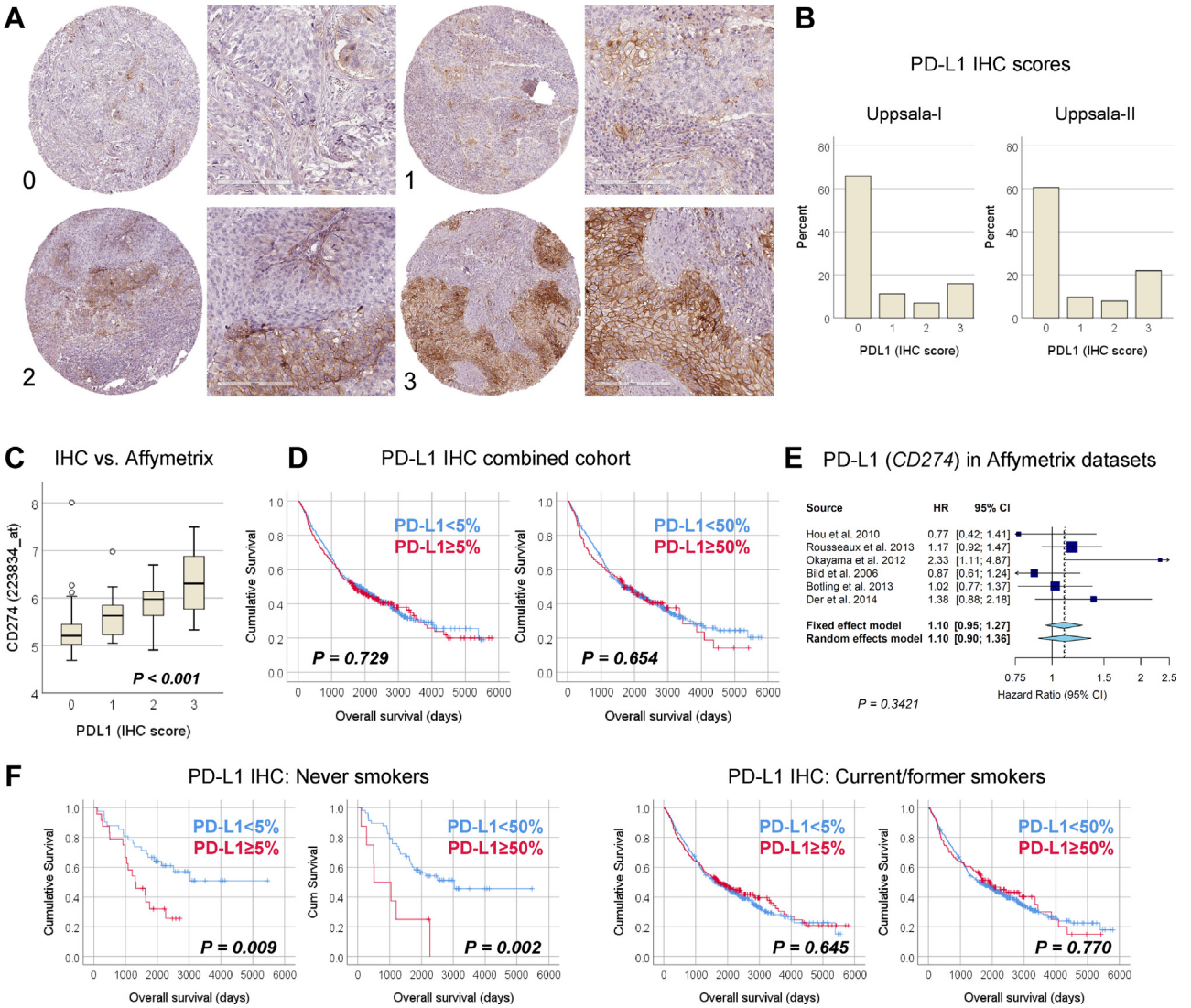

Figure 3. Programmed death ligand 1 (PD-L1) expression in NSCLC. (*A*) Immunohistochemistry (IHC) scores for PD-L1; membranous PD-L1 tumor cell positivity was taken into account. (*B*) Distribution of PD-L1 IHC scores in the Uppsala-I and Uppsala-II tissue microarray (TMA) cohorts. (*C*) PD-L1 IHC score versus *CD274* (Affymetrix probeset 223834_at) transcript level. *p* value from the one-way analysis of variance. (*D*) Kaplan-Meier plots contrasting low versus high IHC scores for PD-L1; IHC score 0 versus 1-3 (cutoff 5%, *left panel*) and IHC score 0-2 versus 3 (cutoff 50%, *right panel*). (*E*) Association of *CD274* expression (223834_at) with overall survival (OS) in a meta-analysis of NSCLC Affymetrix datasets. HR, hazard ratio; 95% CI, 95% confidence interval; *p* value is of the random effects model. (*F*) Association of PD-L1 IHC score (cutoff 5% and 50%) with OS in never-smokers (*left panel*) and current/former smokers (*right panel*).

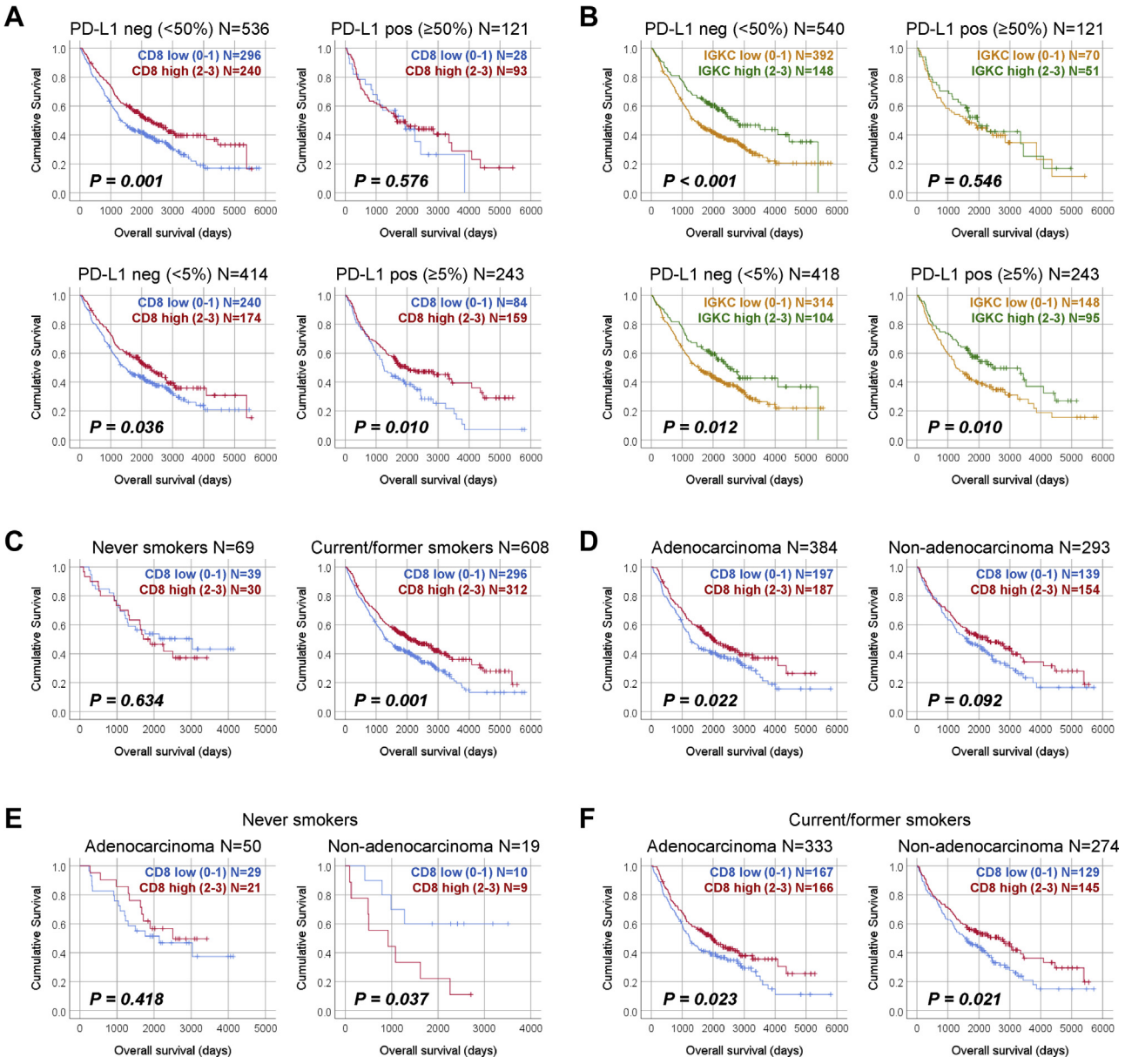

Figure 4. Prognostic impact of cytotoxic T cell (CD8) and plasma cell (IGKC) infiltration stratified by programmed death ligand 1 (PD-L1) status, smoking history, and histology. (*A*) CD8 and overall survival (OS) stratified according to PD-L1 positivity (*upper panel*, cutoff 50%, *lower panel*, cutoff 5%), with CD8 immunohistochemistry (IHC) scores dichotomized as 0-1 versus 2-3. *B*, IGKC and OS stratified according to PD-L1 positivity (*upper panel*, cutoff 50%, *lower panel*, cutoff 5%), with IGKC IHC scores dichotomized as 0-1 versus 2-3. (*C*) Association of the level of CD8-positive T cells with OS in never-smokers (*left*) and current/former smokers (*right*). (*D*) Association of the level of CD8-positive T cells with OS in adenocarcinomas (*left*) and nonadenocarcinomas (*right*). (*E*) Association of the level of CD8-positive T cells with OS in adenocarcinomas (*left*) and nonadenocarcinomas (*right*) of never-smokers. (*F*) Association of the level of CD8-positive T cells with OS in adenocarcinomas (*left*) and nonadenocarcinomas (*right*) of current/former smokers.

collection (N = 1724) of publicly available gene expression microarray datasets. As shown by the corresponding forest plots (Fig. 2*B*), differences between individual NSCLC datasets can, however, be observed, which should be recognized when considering the possible usage of an immuno-score as a prognostic biomarker in NSCLC.

In the present investigation, a higher level of lymphocyte infiltration was found to correlate with PD-L1 positivity, which agrees with previous studies.[14,19] Whereas T cells and plasma cells are mostly reported to be associated with better outcome, the situation for PD-L1 tumor cell positivity in patients who were not treated with PD-1/PD-L1 inhibitors is rather unclear.[13,34,35] Here, no association of PD-L1 with OS was observed in the complete cohort or when the patients were stratified according to the level of lymphocyte infiltration or most

clinicopathologic factors. However, an association with worse prognosis was seen in the never-smoker patient subset. Previous studies have described that PD-L1 positivity was associated with both smoking and never-smoking status.[36,37] In the currently evaluated patient cohort, the fraction of PD-L1–positive tumors did not differ significantly depending on smoking history. Because never-smoking lung cancer represents a small fraction of NSCLCs, the finding that PD-L1 is associated with worse OS in never-smokers should be confirmed in further, independent studies with a sufficient sample size. To explain why PD-L1 is associated with worse OS in never-smokers, but not in current and former smokers, would also require further investigations and was beyond the scope of this study, but it can be speculated that it is related to different mutation rates in smokers and never-smokers. It has been reported that smokers have higher mutation frequencies compared with never-smokers and a higher mutation count per tumor has been shown to correlate with the number of generated neoantigens per tumor, as well as with the estimated cytolytic activity of cytotoxic T cells.[38-40]

We also report that the favorable influence on OS of lymphocyte infiltration was limited to PD-L1–negative tumors, when PD-L1 status was determined based on a cutoff of 50% or greater positive tumor cells (Figs. 4*A* and *B*). It cannot be excluded that the lack of survival association in the PD-L1–positive subset is due to the smaller sample size, but our findings agree with the results of Tokito et al.[41] who showed that the combination of high CD8-positive cell density and the absence of PD-L1 expression was associated with better outcome based on the analysis of 74 stage III NSCLC patients, as well as with findings by Koh et al.[42], who analyzed 497 primarily stage I NSCLCs and showed that PD-L1 negativity in combination with a low PD1/CD8 ratio was associated with longer disease-free survival. On the contrary, a combination of PD-L1 status and level of tumor infiltrating lymphocyte was not associated with survival in the analysis of 170 NSCLCs according to Lin et al.[20] Our results support the conclusion that the presence of immune inhibitory factors should be taken into account if immune cell infiltration is to be considered a prognostic biomarker in NSCLC. Furthermore, additional modifying factors should be considered. The results of the present study propose that PD-L1 tumor expression must exceed a certain quantitative threshold to translate into a loss of the survival benefit which is associated with lymphocyte infiltration; however, the possible influence of the relatively smaller sample size of the PD-L1–positive subset must be noted. Here, PD-L1 positivity in 50% or greater of tumor cells counteracted the survival benefit associated with T and plasma cell infiltration, although this was not observed when PD-L1 positivity was defined by a 5% or greater cutoff (Figs. 4*A* and *B*). Moreover, smoking status appears to be a factor of influence. Lymphocyte infiltration, assessed by the level of CD8- and IGKC-positive cells, did not confer better prognosis in never-smokers (Fig. 4*C*; Supplementary Fig. 5*A*). However, the never-smokers in this cohort were characterized by a significantly lower proportion of highly proliferating tumors ($p < 0.001$, Fisher's exact test) and the positive impact of lymphocyte infiltration is more pronounced in highly proliferative tumors (Figs. 1*C* and *D*). As a final point, the prognostic benefit of CD8-positive lymphocyte infiltration was not observed in never-smoking squamous and large cell lung cancers, but an opposite effect was rather seen (Fig. 4*E*). Nonsmoking-related nonadenocarcinomas may represent a tumor entity that should be considered separately. Taking both histologic subtype and smoking status into consideration is, however, problematic, because the total case number of never-smoking, nonadenocarcinoma patients is low, and these subgroup analyses must be considered as exploratory only, as no adjustment for multiple testing was performed in the present study.

Finally, possible limitations of the present investigation should be acknowledged. First, several anti–PD-L1 antibodies are available and the choice of antibody may influence the results. Here, the rabbit monoclonal anti–PD-L1 antibody clone E1L3N was used. We used a cutoff for positivity of 5% or greater to enable a comparison to one of the cutoffs for the U.S. Food and Drug Administration (FDA)-approved Dako 28-8 pharmDx kit for nivolumab; alternatively we used a cutoff of 50% or greater, as with the FDA-approved Dako 22C3 pharmDx kit for pembrolizumab.[43-45] That corresponding transcript-level information was additionally interrogated and correlated well with the IHC scores for PD-L1 (Fig. 3*C*) as well as for the analyzed immune cell markers (Fig. 2*A*) supports the reliability of the IHC-based evaluation. Second, because PD-L1 is reported to be subject to intratumoral differences, PD-L1 positivity may remain undetected when a TMA is analyzed instead of whole sections, especially when defined by a low cutoff for the percentage of positive tumor cells.[46] Intratumor differences in the pattern of lymphocyte infiltration may also be inadequately represented due to the sampling of the TMA cores. Third, only tumor cell expression was evaluated, whereas PD-L1 is also expressed on various immune cell subsets and it cannot be excluded that PD-L1–positive intratumoral macrophages in some cases were mistakenly annotated as tumor cells. Lastly, assessment of lymphocyte infiltration in the tumor microenvironment is challenging due to intra- and

intertumor differences with regard to the abundance of tumor stroma in relation to the number of tumor cell as well as variations in stromal cellularity and cell type composition. In addition to drawbacks related with the use of IHC and TMAs, the merging of two cohorts collected during different periods may be considered suboptimal, as standard treatments and diagnostic criteria for the histologies have changed over time.

In summary, we show that T cell, as well as B and plasma cell, infiltration is associated with better outcome in NSCLC patients who were not treated with PD-1/PD-L1 inhibitors, and that this association is stronger in highly proliferative tumors. PD-L1 positivity was not associated with OS in the nonstratified NSCLC population. However, we observed a significant association of PD-L1 positivity with shorter OS in never-smokers, which was validated on the RNA level in one independent dataset, but should be confirmed in a sufficiently large population of nonsmoking-related NSCLC. Finally, no prognostic association of lymphocyte infiltration was observed in the PD-L1–positive tumor subset defined by a 50% cutoff. The prognostic impact of lymphocytes also depended on patients' smoking history and histologic subtype. The impact of PD-L1 status, smoking history, and histology should be taken into account, in addition to proliferation, if lymphocyte infiltration is to be considered a prognostic biomarker in NSCLC.

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Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at www.jto.org and at https://doi.org/10.1016/j.jtho.2018.12.022.

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